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13. ABSTRACT *(Maximum 200 Words)*

We propose to establish an approach by which tumor cells are eradicated through selective induction of $CD8^+$ T cells specific for a protein overexpressed in many adenocarcinomas. A peptide derived from *HER2/neu* (HN654-662) stimulates cytotoxic T lymphocytes (CTL) that lyse primary tumors from ovarian or breast cancers. It has been proposed that the poor immunogenicity of HN654-662 is due to poor binding to HLA-A2.1. These data demonstrate HN654-662 is an extremely poor HLA-A2.1 binding peptide. Furthermore, modifications to anchor residues, predicted to improve binding, do not significantly increase affinity. We have determined the structure of A2/HN654-662 and show that the center of the peptide is disordered. Most substitutions designed to increase affinity do not do so. Individual substitutions at positions 3, 5, 6 and 7 do not significantly increase affinity. Multiple substitutions in the peptide, that individually increase affinity, actually decrease affinity to HLA-A2.1. $A2K^b$ mice do not generate a response to HN654-662 either as peptide or bound to dendritic cells. Some of the variants of HN654-662 do generate a CTL response. $A2K^b$ x neu mice generate spontaneous tumors around 5 months of age. These mice can be induced to generate a CTL response against peptides presented by the tumor.

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FOREWORD

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5. Introduction

Cytotoxic T lymphocyte (CTL) responses to tumors in both man and animal models have been demonstrated [1-4]. Tumor cells are autologous and should be immunologically ignored, but many are recognized. These tumor cells may express mutated proteins, new proteins encoded by transforming viruses, or proteins normally expressed at low levels, but now are expressed at high levels. The primary discriminator of T cell activation is the interaction of the T cell receptor (TcR) and the MHC molecule. It is this interaction that the goals of this proposal seek to enhance.

Class I MHC molecules are ternary complexes found on the plasma membrane of nearly all cells in the body. These molecules contain a polymorphic heavy chain, β_2-microglobulin (β_2m) and a small peptide (typically 8-10 amino acids). The heavy chains are synthesized, co-translationally translocated into the endoplasmic reticulum (ER), associate with the molecular chaperones calnexin and calreticulin and with peptide-loading molecules such as p48 and TAP [5]. Only complexes that are completely assembled with peptide and β_2m are allowed to egress from the ER into the Golgi [6]. The availability of peptide appears to be the rate-limiting step in cell surface expression of class I proteins [7].

An analysis of peptides that bind to specific class I molecules show some positions in the peptide that are relatively invariant [8, 9]. These amino acids interact with substructures of the MHC molecule called pockets [10]. Originally the anchor side chains were believed to provide the majority of the free energy of binding. Subsequent analyses by our laboratories and others have clearly demonstrated that a peptide's binding ability depends on positive and negative effects from all residues within the peptide [11-13]. Possession of amino acid side chains, which would be favorable anchors, is not sufficient to make a peptide bind; nor is the absence of the residues sufficient to render a peptide unbindable. Indeed, many peptides, which seem as likely to fit, do not function as epitopes to T cells *in vivo* [14] suggesting that they do not bind to the class I molecules.

The issue of peptides binding to class I molecules is important since peptides which are <u>not</u> bound to class I and are <u>not</u> present on the surface, are <u>not</u> immunogenic. However, the exact effect of the affinity of peptide for class I, the stability of the complex on the cell surface and its subsequent immunogenicity is a matter of conjecture at the present time.

This interaction between heavy chain and peptide is crucial not only in the generation of CTL responses, but also in thymic selection. Class I MHC/peptide complexes are required for the egress of mature T cells from the thymus and are important in both positive and negative selection during T cell development [15, 16]. Recent experiments have shown that during thymic education the fate of the T cell (deletion or proliferation) is dependent on the affinity of the class I MHC/peptide complex and the T cell receptor [17]. Therefore, the affinity between TCR and class I MHC/peptide complex is critical for function.

A *HER2/neu* derived peptide has been identified that is recognized by autologous CTL [18]. This peptide HN654-662 (IISAVVGIL), has the HLA-A2.1 binding motif [8] and has been shown to stimulate CTL from tumor infiltrating lymphocytes derived from breast and ovarian tumors [19]. However, CTLs stimulated by HN654-662 exhibit poor cytotoxicity possibly due to the peptides poor solubility and poor binding affinity. To gain further insight into the factors that govern CTL activity, we examined the binding of HN654-662 to recombinant HLA-A2.1. As seen in our preliminary results, this peptide is extremely unusual for a peptide that stimulates CTL activity. HN654-662 marginally binds HLA-A2.1 and modifications shown to increase the

affinity of other peptides ([20] and unpublished data) have little effect. Therefore, this peptide gives us the rare opportunity to use structural biology as a tool to solve an important biological problem in a timely fashion. The crystallographic structure of HN654-662 will provide information to explain the poor binding of the peptide.

Structural biology gives insights into function/importance that are not apparent from other data. For example, groups have identified peptides that do not appear to bind to class I MHC molecules in the usual manner [21]. It was the crystal structure of a HLA/peptide complex that demonstrated that the peptide extended out of the carboxyl terminus [22]. Recent advances in technology have increased the speed at which structures may be determined to the extent that structural biology is now a useful tool to probe function. For example, a class I histocompatibility complex structure can go from an idea to a finished structure solved by molecular replacement in 2-3 months. We believe our extensive experience in the biophysical studies of class I MHC/peptide interactions and class I MHC mediated CTL killing will allow us to enter a new field, cancer immunotherapy, and make significant contributions.

6. Experimental Methods (Derived From Statement of Work)

Specific Aim 1. Develop HN654-662 variant peptides with improved affinity for HLA-A2.1

A The first task is to determine the co-crystal structure of HLA-A2.1 complexed with wild-type HN654-662 peptide. Objective: Complete by 12/96.

Methods: A soluble recombinant form of HLA-A2 is folded in vitro in the presence of β2m and HN654-662. The protein is purified by gel filtration chromatography. It is concentrated to 10 mg/ml and buffer exchanged to 25 mM MES pH6.5 for crystallization trials. Initial crystals that form are crushed to make seed crystals for additional trails. Large single crystals are transferred to cryoprotectant and rapidly cool to -180°C by plunging into liquid propane. The crystals are stored as solid propane popsicles in liquid nitrogen until use. Crystallographic data are collected and the structure determined by molecular replacement methods.

Progress: We have finished the structure of HN654-662 bound to A2 and have just had the manuscript approved for publication in The Journal of Biological Chemistry. A copy of the manuscript is enclosed as Appendix I. The structure shows that the peptide does not bind well because the center of the peptide does not make stabilizing contacts with the MHC peptide-binding groove.

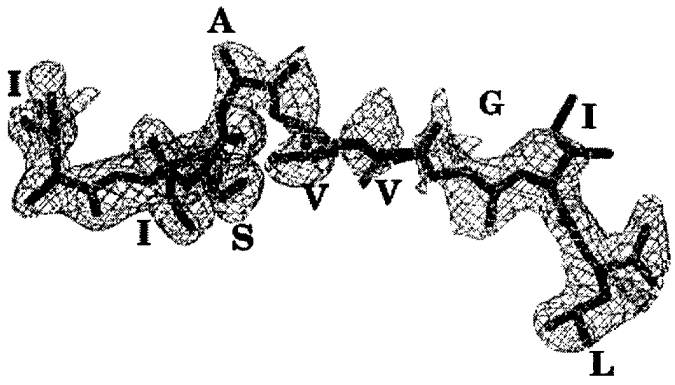


FIGURE 1. The center of the HN654-662 peptide is disordered. The averaged omit electron density map of the HN654-662 peptide with a cover radius of 1.5 Å. A2 has been removed for clarity. For more details see our manuscript attached as an appendix.

B Using the crystal structure, identify an amino acid that points down into the peptide binding cleft. Synthesize

a peptide library with 20 different peptides. Fold HLA-A2 with library and isolate stabilizing peptides. Identify residue that points up towards TCR. Complete by 2/97

Methods: The library may be synthesized using standard FMOC chemistry on a solid phase synthesizer. The position to be randomized is coupled with a mixture of 19 amino acids (cysteine left out to reduce difficulties in the folding reaction). The difficulty with the library is that salts, etc that cannot be effectively purified away inhibit folding of our protein. However, the library may be added to the folding cocktail of A2 and protein isolated as described. Folded protein will contain those peptides that allow for productive complexes. The peptides may be isolated after treatment by spinning through a centricon-3 filter apparatus (Amicon) and identified by mass spectrometry.

Progress: In the absence of the crystal structure, we had produced one library randomizing position 3. Position 3 has been shown to be a secondary anchor in many peptides. As the peptide only has one polar residue and it is the serine at P3, we decided to substitute the first position from isoleucine to lysine to improve solubility of the peptide library. The library was used in our *in vitro* folding reaction and large quantities of A2 were isolated. The protein is not very stable however. Warming the mixture to room temperature resulted in complete denaturation of the complex. We repeated the experiment keeping the preparation cool at all times. The protein was sent to collaborators at Zycos Inc who are very talented mass spectroscopists. Based on the mass data, all of the 19 amino acids in the library were present bound to A2. Since mass spectrometry is not quantitative, it suggests that this technique is not viable for this goal. We intend to repeat the experiment and sequence the eluted peptides by Edman degradation. This will give us a quantitative measurement of the frequency of each amino acid at position 3.

With the crystal structure in hand, we have found that position five points mostly sideways (Figure 2). The remaining residues within the cleft are not well defined (as described above and in the conclusions) and we are unable to predict which residues should be pointing down and which up. A substitution of valine at position five with leucine results in a peptide with higher affinity (Figure 3). The residues that do not appear to be important for TcR recognition will be our next targets for library formation. We have not attempted this yet because we have been unable to design peptides that bind with high affinity (see C below).

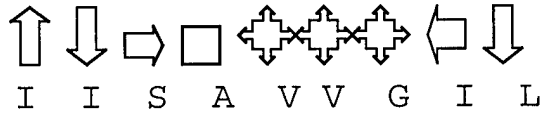


Figure 2. Orientations of the amino acids of H654-662 when bound to HLA-A2. Arrows pointing in all four directions: we cannot reliably position these amino acids; Up arrow: towards TcR; Down arrow: towards beta pleated sheet; Right arrow: towards α2 α helix; Left arrow: towards α1 α helix.

C Chemically synthesize peptides that improve stability and determine thermostability. Complete by 4/97.

Progress: We hypothesized that the central residues in HN654-662 were not conducive to forming stable interactions with the A2 binding cleft. Therefore, in order not to bias the study, we synthesized a variety of ligands with substitutions of HN654-662 at positions 5-7 and tested

for binding affinity. We measured a relative binding constant by adding peptide exogenously to T2 cells that lack a functional peptide transporter. A2 on the surface of these cells are relatively peptide receptive and by virtue of the peptide binding, the A2 is stabilized on the cell surface. The amount of A2 on the surface can be followed with antibodies and the quantified by flow cytometry.

As can be seen from Table I, we were totally wrong in our hypothesis. Most of the substitutions did not improve affinity. The only one that did is V5L and we can explain that from the crystallographic structure.

Name	Sequence	K_r	Name	Sequence	K_r	Name	Sequence	K_r
V5F	IISA**F**VGIL	>50	V6D	IISAV**D**GIL	>50	G7A	IISAVV**A**IL	ND
V5L	IISA**L**VGIL	11.1	V6F	IISAV**F**GIL	>50	G7D	IISAVV**D**IL	>50
V5G	IISA**G**VGIL	>50	V6G	IISAV**G**GIL	>50	G7F	IISAVV**F**IL	>50
V5D	IISA**D**VGIL	>50	V6K	IISAV**K**GIL	>50	G7K	IISAVV**K**IL	>50
V5K	IISA**K**VGIL	>50	V6T	IISAV**T**GIL	>50	G7T	IISAVV**T**IL	ND
ML	MLLSVPLLL	1.8				HN654-662	IISAVVGIL	>50

Table I. Most substitutions at P5, P6 and P7 of HN654-662 do not increase binding affinity. K_r is the relative binding constant determined by a T2 cell surface assembly assay. K_r is defined to be the concentration of peptide in μM that yields 50% mean channel fluorescence as compared to the ML peptide. ND not determined. Most substitutions at P5 (V5D, V5G, V5K, and V5T) bind so poorly that even at 50 μM their fluorescence is below the no peptide controls.

As a result, we developed a new hypothesis. This stated that the position 3 residue, which is a secondary anchor, is too small in A2 (it is a serine). We decided to make as series of substitutions at P3 to increase affinity. Once again, we were totally wrong (Table II).

Name	**Sequence**	**K_r**	**MF**
S3	KISAVVGIL	>50	31.2
S3D	KIDAVVGIL	>50	8.4
S3F	KIFAVVGIL	>50	27.8
S3G	KIGAVVGIL	>50	20.2
S3L	KILAVVGIL	>50	9.6
S3T	KITAVVGIL	>50	9.7

Table II. **P3 substitutions do not improve binding affinity**. Description of each is as described in the legend to Table I. MF is maximal fluorescence. This shows that although all the peptides were of relatively low affinity, we could observe differences. Surprisingly, the best appeared to be the wild-type serine at position three.

Single positions don't make high affinity peptides regardless of the position or the identity. Therefore, we come back to our first hypothesis that there are combinations of residues that are bad for HN654-662 binding. To reduce the number of peptides to synthesize, we took advantage of the fact that the KIFAVVGIL peptide in the P3 substitution experiment (Table II) is very similar to KIFGSLAFL the E75 peptide from HER-2/neu that binds with much higher affinity. Therefore, we have ordered the following series of peptides in Table IV. This shows a progress from single (2-5), double (6-11) and triple (12-15) amino acids substitutions until the peptide is converted from HN654-662 (S3F) (1) to E75 (16).

	Sequence
1	KIFAVVGIL
2	KIF**G**VVGIL
3	KIFA**S**VGIL
4	KIFAV**L**GIL
5	KIFAVV**A**IL
6	KIF**GS**VGIL
7	KIFA**SL**GIL
8	KIFAV**LA**IL
9	KIF**GVVA**IL
10	KIF**GVLG**IL
11	KIFA**SVA**IL
12	KIF**GSLG**IL
13	KIFA**SLA**IL
14	KIF**GSVA**IL
15	KIF**GVLA**IL
16	KIF**GSLAF**L

Table IV. Factorial substitutions from HN654-662 to E75. The progressive changes are shown in bold-faced type. Position eight in the peptide points up into solvent and therefore was not considered as a variable in this series of peptides.

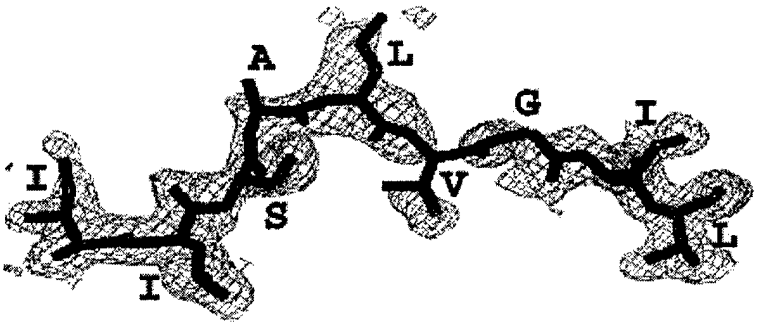


Figure 2. **The structure of a triple-substituted, HN654-662-variant peptide, I2L/V5L/L9V, has broken electron density, as does HN654-662.** Averaged omit density (DM) is shown with a 1.5 Å cover radius at a contour of 1 sigma. The A2 molecule has been removed for clarity. The peptide, ILSALVGIV, was folded with A2 *in vitro*, purified and crystallized as described previously (9, 25). The crystallographic data were collected in 2° oscillations on an R-Axis IIC imaging plate system. The data were processed with DENZO and SCALEPACK and the molecular replacement solution determined by AMoRe. Refinement was conducted with CNS. The structure is completed with good statistics.

We have also made the following observation. Individual substitutions such as I2L, L9V or V5L all increase binding affinity. However, combinations of these substitutions were not additive (Table III). This result is unanticipated as it has been reported that each position is independent (31). We are looking into the structural basis of this now. We believe that the increased binding affinity of V5L is because the leucine binds in a nonpolar pocket under the α2 α helix (Fig 2). However, the structure of the triple substitution, I2L/V5L/L9V (ILSALVGIV), shows the position 5 leucine directed towards solvent. This does not make sense for the single substitution of V5L. We are determining the structures of V5L, I2L/L9V, I2L/V5L, V5L/L9V now.

Name	Sequence	K_r (μM)	T_m (°C)
HN654-662	IISAVVGIL	>50	36.4
I2L	ILSAVVGIL	22.9	42.2
L9V	IISAVVGIV	>50	38.8
I2L/L9V	ILSAVVGIV	10.0	42.5
V5L	IISALVGIL	11.1	45.8
I2L/V5L	ILSALVGIL	49.3	39.0
V5L/L9V	IISALVGIV	>50	38.8
I2L/V5L/L9V	ILSALVGIV	17.5	39.5

Table III. Affinity measurements for combinations of substitutions show that the stabilizations are not additive. T_m values are the temperature at which 50% of the protein is denatured. Kr is defined in Table I.

D. *Chemically synthesize cysteine mutant sequence and test for binding to HLA-A2 in an in vitro assay. Link to Biacore chip and measure on and off-rates to HLA-A2.1. Complete by 9/97.*

Methods: We will synthesize the peptides through standard FMOC chemistry and will test them for binding as described above.

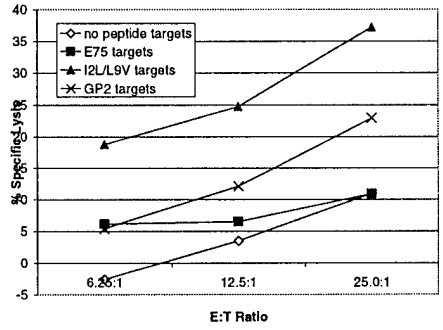
Progress: We have not begun this aim. We have instead focused on determining why HN654-662 binds poorly and dissecting the interactions between the binding residues.

Specific Aim 2. Screen improved epitopes for enhanced affinity for the T cell receptor.

A. Isolate murine CTL lines specific for HN654-662. Line by 12/96 clone by 6/97. Approx. 75 mice.

Methods: CTL are most readily made by stimulation by professional antigen-presenting cells. We will isolate dendritic cells from PBLs of $A2K^b$ mice and stimulate T cells with HN654-662 and altered-peptide ligands. Lines will be generated by repeated in vitro stimulations, clones by limiting dilution or FACS.

Figure 4. **I2L variants are immunogenic in $A2K^b$ mice**. Mice were immunized as above with I2L/L9V and CTL measured as described above. As can be seen the I2L/L9V immunized mice recognize I2L/L9V pulsed targets significantly better than E75 pulsed peptide (irrelevant peptide), HN654-662 or no peptide targets.



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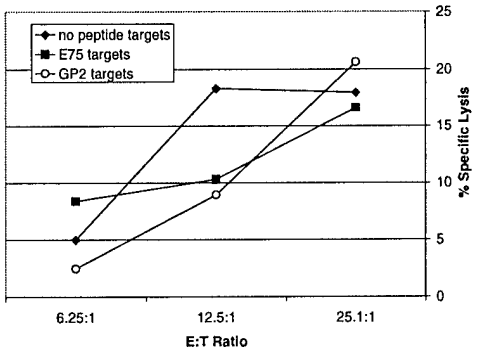

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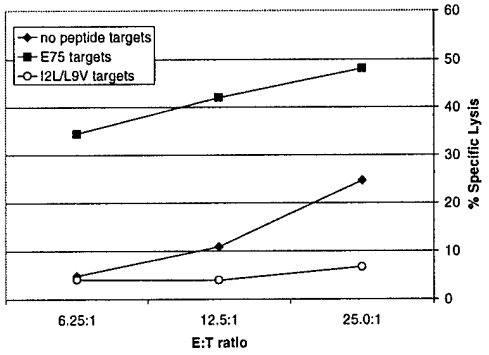

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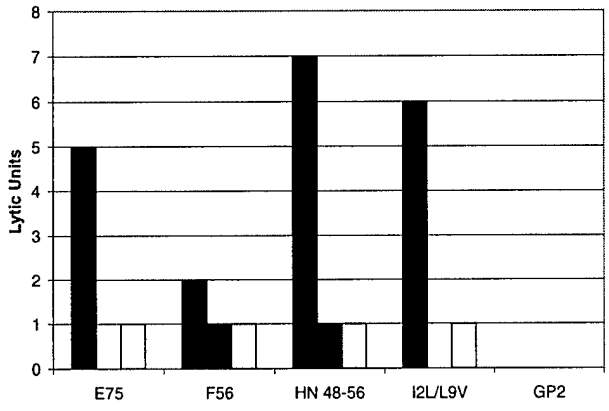

Figure 3. **HN654-662 is not immunogenic in A2K^b transgenic mice, but other HER-2/neu-derived peptides are.** A. Titration of effector cells shows that the effectors generated by HN654-662 immunization do not recognize HN654-662 better than E75. B. E75 immunized mice show a good specific response. However, another HER-2/neu-derived peptide with similarly poor affinity, F56 (YISAWPDSL), is immunogenic. A variant of HN654-662 with slightly higher affinity, I2L/L9V is also immunogenic. Higher affinity HER-2/neu derived peptides E75 and HN48-56 are also immunogenic. A2K^b mice were immunized 3 times in 10-day intervals after which spleens and popliteal and inguinal lymph nodes were removed. The cells were stimulated with irradiated splenocytes peptide pulsed with the peptides given on the x-axis. C. Lysis data converted to lytic units to give one value describing the number of effectors per 10^6 cells.

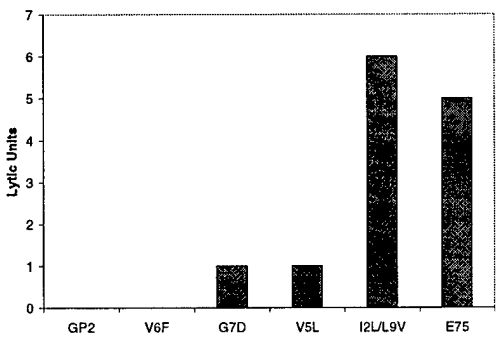

Figure 5. **HN654-662 variants at positions 5, 6 and 7 are not immunogenic compared to anchor variants or other HER-2/neu peptides.** Immunizations and CTL lysis are as described above. Lytic units are the regression analysis of %specific lysis versus E:T ratio. The number is the number of effectors per 10^6 cells that generate 20% specific lysis.

Progress: We used human PBLs to generate HN654-662 specific T cells. HLA-A2 donors were obtained from a different project. Dendritic cells from these donors were used to stimulate T cells using HN654-662 (HN654-662). A very weak response to HN654-662 was generated after three stimulations in vitro with human dendritic cells, but it was not readily reproducible

and the level of specificity was not high. The T cells did not survive long enough to allow us to clone these cells either.

Dr. Roland Tisch (UNC Microbiology and Immunology) has made mice transgenic for a chimeric A2K^b molecule. We have immunized these mice with HN654-662 and a set of variants to test for immunogenicity. HN654-662 failed to generate a response in these mice regardless of the immunization protocol. In the past year we have immunized with a variety of variant peptides. The question we wished to ask was whether the flexibility in the center of the peptide observed in the crystal structure was detrimental to immunogenicity. To investigate this idea, we are immunizing with some of the variant peptides in Table I that contain the wild-type anchors. We have found that some of these peptides generate an immune response, but it is very weak (Figure 4). The poor immunogenicity is not directly related to the poor affinity because another HER-2/neu peptide F56, is immunogenic. We will then make the same substitutions with higher affinity anchor (I2L) and test for immunogenicity again.

B. Transfect LINE1 with HER2/neu and HLA-A2.1 and select. Complete by 12/96
Methods: Using cDNAs for HER-2/neu and HLA-A2/K^b, make stable transformants of LINE1 for inoculation into the Tg A2/K^b mice.

Progress: We have altered the project to examine why the HN654-662 peptide is not immunogenic in mice and looking at other immunogenic peptides from HER-2/neu. In addition, we do not need to transfect LINE1 because A2Kb x neu mice spontaneously generate tumors. We are culturing those tumors now (See Section F).

C. Test CTL reactivity of variant peptide determined from 1 using CTL clones. Complete by 12/97
Methods: Using RMA-S cells or syngeneic B cells, peptide pulse and perform the standard 4 hour chromium release assay on APL.

Progress: As described above, we have switched our efforts to examining why HN654-662 does not bind well and why it is not immunogenic. We now have the A2K^b mouse. We cannot however generate T cells towards HN654-662. If we are able to generate a good response with one of the variants that recognizes HN654-662 also, we will clone those T cells and perform this aim.
Methods: We could not previously use the mice of choice, the mice that are transgenic for HLA-A2/K^b are presently on the wrong genetic background. We had altered our plans to use human T cells and test reactivity against established tumor cell lines and primary tumors. However, we were not successful at reproducibly generating a response to HN654-662 from human PBMC. Since we now have the mice required, and the question has shifted slightly, we are again working in the A2K^b mice on the FVB background. A2K^b mice were immunized with the peptide substitutions shown in Table I and the immune response has and continues to be tested. Figure 1 shows that responses can be made to I2L/L9V, but not HN654-662 or V5L suggesting that the affinity of HN654-662 is just too low to generate a primary immune response. However, an equally poor binding HER-2/neu derived peptide (F56) is able to generate a good response.
E. Produce soluble TCR. Complete by 6/98.
Methods: Isolate cDNA using Fast-Track kit (Invitrogen). Clone and determine sequence. Using PCR make constructs for expression in the baculovirus system and also as fusion proteins of variable domains using the TrcThioHis system (Invitrogen). Isolate by metal chelation

chromatography and other chromatographic methods as required. Test for proper folding using monoclonal antibodies that recognize the corresponding alpha and beta chains.
Progress: We have put this aim on hold until we identify an APL that generates a good response to HN654-662.

F. Test affinities of class I/ peptide complexes with soluble TCR. Complete by 12/98.
Methods: Surface plasmon resonance will be used to measure on and off rates of complexes fixed to the surface of appropriate chips. In each case, we will engineer E. coli BirA recognition sites to specifically biotinylate the carboxyl terminus of each protein. Then each protein individually will be bound to strepavidin-coated chips. On and off rates should be independent of which protein is coupled to the chip.

Progress: We have successfully engineered BirA recognition sequences to the carboxyl end of HLA-A2.1. These proteins fold in vitro as well as the wild-type A2 sequences. We will perform the same to the T cell clone isolated. We have also successfully cloned a different TcR and testing our abilities to produce the protein when we get a HN654-662-specific T cell clone.

G. Test class I peptides identified in 1 as vaccines with transgenic mice. Complete by end of grant.
Methods: Peptide-pulsed dendritic cells (DCs) will be used as vaccines to generate specific T cell responses. The DCs may be used to test protection in a tumor challenge model or tested for their ability to slow or reduce the growth of a tumor.

Progress: In collaboration with Dr. Roland Tisch, we now have a good tumor challenge model. We have crossed the $A2K^b$ mice onto the rat HER-2/neu tg mouse to get an $A2K^b$ x neu mouse. The rat neu gene is under control of a mouse mammary tumor virus promoter. These mice spontaneously develop mammary tumors at approximately 5 months of age. We have begun to examine the T cell response to HER-2/neu peptides bound to A2Kb. Unfortunately, HN654-662 is not common between human and rat protein sequences so we cannot use this model system to study HN654-662. We are using to study other low affinity peptides from HER-2/neu that are common between the human and rat proteins. We are able to generate a good CTL response from many of these peptides. We do not know if these responses will be protective at this time. We are culturing the tumors to generate tumor lines that may be used in a tumor challenge situation.

7. Key Research Accomplishments

- Determined crystallographic structure of A2/HN654-662
- Measured binding to large numbers of peptide variants that do not significantly increase affinity.
- Determined structure of A2/I2L/V5L/L9V
- Designed peptides with increased affinity that when used to immunize mice generate CTL that react with HN654-662.
- Measured CTL responses from $A2K^b$ x neu mice to low and intermediate affinity peptides presented by the tumors. This suggests that developing anti-tumor CTL is a viable technique.

8. Reportable Outcomes

Manuscripts:

Kuhns, J. J., Batalia, M. A., Yan, S., and Collins, E. J., Poor Binding of HER2/neu Epitope to HLA-A2.1 is Due to Lack of Interactions in the Center of the Peptide, *J. Biol. Chem., in press* (1999).

Applied for NIH R01 (10/99) based on this work.

9. Conclusions:

We have made significant progress within the last year. We have had one manuscript accepted and another is out for review and two others that have experiments nearly completed. We have used that information to design peptides that have improved binding to class I MHC. We have tested many more that do not have increased binding affinity. We are close to understanding the poor binding affinity of HN654-662. We have found a new phenomenon of interaction between the binding residues in the peptide. We have a good animal model to test for immunogenicity of these peptides. We have redesigned the experimental scheme to examine T cell recognition in transgenic mice. We now have all the tools at hand and the experiments are in full swing. We expect the next year to be even more fruitful than this past year.

10. References

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11. Appendices: none